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Formation of IL-7Rα^{hi} and IL-7Rα^{lo} CD8 T cells during infection is regulated by the opposing functions of GABPα and Gfi-1

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Abstract

IL-7 is essential for survival of naïve and memory T cells, and IL-7 receptor alpha chain (IL-7Rα) expression is dynamically regulated in activated CD8 T cells during acute viral and bacterial infections. Most virus-specific CD8 T cells become IL-7Rα^{lo} and are relatively short-lived, but some escape IL-7Rα repression (referred to as IL-7Rα^{hi} memory precursor effector cells (MPECs)) and preferentially enter the memory CD8 T cell pool. How anti-viral effector CD8 T cells regulate IL-7Rα expression in an “on and off” fashion remains to be characterized. During LCMV infection, we found that opposing actions of the transcription factors GABPα and Gfi-1 control IL-7Rα expression in effector CD8 T cells. Specifically, GABPα was required for IL-7Rα expression in MPECs and this correlated with hyperacetylation of the *Il7ra* promoter. On the other hand, Gfi-1 was required for stable IL-7Rα repression in effector CD8 T cells and acted by antagonizing GABPα binding and recruiting HDAC1, which deacetylated the *Il7ra* promoter. Thus, *Il7ra* promoter acetylation and activity was dependent on the reciprocal binding of GABPα and Gfi-1, and these data provide a biochemical mechanism for the generation stable IL-7Rα^{hi} and IL-7Rα^{lo} states in virus-specific effector CD8 T cells.

Keywords

IL-7Rα; GABPα; Gfi-1; LCMV; memory T cells; CD8 T cells

Introduction

Interleukin-7 (IL-7) plays an essential role in lymphocyte development and survival and has been shown to be a key regulator of memory T cell homeostasis (1,2). IL-7 signals are transduced through the high-affinity IL-7 receptor alpha chain (IL-7Rα) and the common cytokine receptor gamma chain (γ_c; CD132) (1,3). IL-7R signals downstream through at least two major signal transduction pathways, the JAK/STAT and PI3K/AKT pathways, which together induce expression of several genes that promote T cell growth and proliferation and survival (4–7).

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The expression of IL-7Rα is dynamically controlled at many different stages during the life of T and B cells. For example, in the development of mature naïve B and T cells, IL-7Rα is expressed by common lymphoid progenitors (CLPs), but then is downregulated during the transition from pre-B to immature and mature B cells (8). During thymocyte development, IL-7Rα is expressed by double negative (DN) 1 and 2 cells, then repressed as they progress to the double positive (DP) stage, and finally, it is re-expressed on single positive (SP) mature T cells and maintained on naïve CD4 and CD8 T cells in the periphery (9,10). Survival of mature naïve T cells as they circulate between blood and secondary lymphoid organs requires IL-7:IL-7Rα signaling (1,2).

When a naïve T cell encounters antigen during infection, and enters a new phase of differentiation, IL-7Rα is downregulated once again as the activated T cells clonally expand and differentiate into effector T cells (11–14). Several reports have shown that during acute infections in mice, such as lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes*, the majority of the antigen-specific CD8 T cells express low levels of IL-7Rα (referred to as IL-7Rα^{lo}) at the peak of expansion (11,12,14,15). However, a small subset of the effector CD8 T cells express higher amounts of IL-7Rα (referred to as IL-7Rα^{hi}), and these two subsets have been shown to have different cell fates (12,14). The IL-7Rα^{hi} effector CD8 T cells have been considered memory precursor effector cells (MPECs) because they preferentially survive and develop into a stable long-lived memory CD8 T cell population that can protect against secondary infection and self-renew through homeostatic turnover (12,16). These MPECs require functional IL-7Rα to become long-lived memory CD8 T cells (2,12,17), and a similar process likely occurs in memory CD4 T cell development (18,19). In contrast, the IL-7Rα^{lo} CD8 T cell subset is relatively short-lived and only poorly forms memory T cells (12,16). The majority of the antiviral IL-7Rα^{lo} effector cells do not appear to convert to an IL-7Rα^{hi} state, indicating stable repression of the IL-7Rα locus is maintained over time in the absence of antigenic stimulation (20). Although it is likely that the steady IL-7 deprivation contributes the finite lifespan of IL-7Rα^{lo} cells, recent data over expressing IL-7Rα on these cells shows that IL-7Rα downregulation is symptomatic of, but not causal to the bulk effector cell death that occurs after viral clearance (21). These IL-7Rα^{lo} cells become dependent on IL-15, but this does not sustain them long-term (20).

Considering that fairly stable changes in IL-7Rα expression accompany the decision to become a memory T cell or not, and that memory T cell longevity is functionally dependent on IL-7Rα expression, it is important to understand how IL-7Rα expression is regulated in antigen-specific T cells during immune responses. Currently, most evidence suggests that control of IL-7Rα expression in T cells is mediated primarily through changes in *Il7ra* gene transcription (12,13,22,23), but very few of the mechanistic details of this process are known. Multiple signals can affect *Il7ra* transcription in T cells including IL-7 (23), IL-2 (24) and TCR signaling that repress (13,25), and glucocorticoids (13,26) that induce *Il7ra* transcription. In addition, a few transcription factors have been identified that directly bind to and control *Il7ra* expression. Two transcription factors (TFs) of the ets family, PU.1 and GA binding protein alpha (GABPα; also known as (*aka*) NRF-2 and E4TF1-60), bind to the same site in the *Il7ra* promoter and activate its transcription(27–29). GABPαis part of a heterodimeric complex that includes GABPβ (*aka* NRF1) that can activate transcription and recruit histone acetyltransferases (HATs) (30). In B cell progenitors, both GABP and PU.1 drive IL-7Rα expression (28,31), but in thymocytes and naïve T cells GABP alone performs this function (29). The reason behind the separation of functions of PU.1 and GABP in the different lymphocyte lineages is not clear. Moreover, it is not known if GABP functions in memory T cells or their precursors to maintain IL-7Rα expression. To date, growth factor independence 1 (Gfi-1) is the only known IL-7Rα transcriptional repressor in T cells; it binds to introns 2 and 4 of the *Il7ra* gene and downregulates expression following IL-7 signaling(23). Gfi-1 has been shown to recruit G9a histone lysine methylase, ETO proteins and histone deacetylases

(HDACs), to mediate transcriptional repression of other genes (32). Whether Gfi-1 acts at other stages of T cell development or following TCR activation to silence the IL-7Rα locus remains to be determined.

The aims of this study were to determine how *Il7ra* is transcriptionally regulated in antigen-specific effector and memory CD8 T cells following LCMV infection in mice. In particular, we examined if epigenetic modifications of chromatin and the binding of GABPα or Gfi-1 were associated with different transcriptional states of *Il7ra* in effector and memory CD8 T cells. Our data suggests that Gfi-1 and GABPα function in a "ying-yang" relationship during effector CD8 T cell development. Gfi-1 is necessary and sufficient to maintain hypoacetylation of histones and stable *Il7ra* repression in late-stage effector CD8 T cells. Conversely, GABPα helps to maintain histone acetylation and *Il7ra* transcription in IL-7Rα^{hi} MPECs. Together, these data provide a mechanistic outline of *Il7ra* transcriptional regulation as effector and memory CD8 T cells differentiate during viral infection.

Results

Transcriptional repression of Il7ra in LCMV-specific effector CD8 T cells is associated with histone deacetylation and HDAC recruitment

To more thoroughly characterize the kinetics and mechanism(s) of *Il7ra* repression during effector CD8 T cell differentiation, we first measured IL-7Rα protein and mRNA levels as well as histone acetylation and HDAC binding to the *Il7ra* promoter region (from bps −60 thru −295) (Fig. 1A). Two experimental systems, employing endogenous or P14 TCR transgenic (tg) LCMV-specific CD8 T cells, were used to measure effector CD8 T cell IL-7Rα expression as they clonally expand and differentiate in response to LCMV infection. The use of P14 CD8 T cells facilitated earlier detection and isolation of large numbers of the LCMV-specific CD8 T cells; for these experiments, a small number of congenically-marked Thy1.1+ naïve P14 CD8 T cells (<10,000 cells), specific for LCMV epitope D^{b}GP33-41, were adoptively transferred into C57BL/6 mice (Thy1.2+) that were subsequently infected with LCMV.

The surface expression of IL-7Rα was measured using flow cytometry and the gates for IL-7Rα^{hi} and IL-7Rα^{lo} T cells were created based on two criteria: (1) the IL-7Rα staining pattern of total lymphocytes because the two populations of IL-7Rα^{hi} and IL-7Rα^{lo} cells were clearly identifiable (Fig. 1B, top row, left plot), and (2) the treatment of effector CD8 T cells with IL-7 because this treatment virtually abolishes detection of IL-7Rα on T cells (23). (Fig. 1B, top row, right plot). Naive CD8 T cells are for the most part uniformly IL-7Rα^{hi} (median fluorescence intensity (MFI)=50±5), but within ~4 days of infection IL-7Rα levels dropped considerably on most of the activated CD8 T cells in the spleen (Fig. 1B, bottom row). Both the frequency and number of IL-7Rα^{lo} cells steadily increased with each passing day of infection, until day 7–8 post infection (pi) when virus was cleared and effector CD8 T cell expansion peaked. Although a population of IL-7Rα^{hi} cells was consistently present at each time point (based on staining after IL-7 treatment), the IL-7Rα^{hi} effector cell population became more discernable by day 8 pi (Fig. 1B, bottom row). Previous studies have shown that these IL-7Rα^{hi} effector CD8 T cells present at day 8 pi preferentially develop into long-lived memory CD8 T cells, and hence, are referred to as memory precursor effector cells (MPECs) (12,20). Consequently, the majority of memory P14 CD8 T cells found at days 40+ pi were IL-7Rα^{hi} (12) (Fig 1B, bottom row). Although the proportion of IL-7Rα^{lo} effector CD8 T cells was larger in the lung and smaller in the iLN relative to the spleen, liver and blood, the overall kinetics of IL-7Rα repression on effector CD8 T cells occurred similarly in these tissues, indicating that the formation of IL-7Rα^{lo} CD8 T cells was not compartmentalized to a particular tissue (data not shown).

To determine if a decrease in IL-7Rα mRNA mirrored the reduction of surface IL-7Rα expression, the P14 CD8 T cells were purified at days 5 and 8 pi by FACS and IL-7Rα mRNA levels were measured using quantitative real-time PCR (qRT-PCR). Compared to naïve CD8 T cells, IL-7Rα mRNA levels dropped ~5–9 fold in the effector CD8 T cells, consistent with significant transcriptional repression in these cells (Fig.1C). Further comparison of day 8 effector CD8 T cells that were separated based on IL-7Rα^{hi} and IL-7Rα^{lo} expression revealed that the IL-7Rα^{hi} cells contained ~41 fold more IL-7Rα mRNA than IL-7Rα^{lo} cells (Fig.1C). Also, the mRNA expression of IL-7Rα^{hi} CD8 T cells was comparable to memory CD8 T cells. This indicated that IL-7Rα mRNA levels directly correlate with surface protein levels, and that for the most part, IL-7Rα downregulation during effector cell development is transcriptionally regulated, as has been previously shown in activated T cells both in vivo and in vitro (12,13).

Next, we determined if the repression of IL-7Rα was associated with chromatin modifications such as deacetylation of histone amino-terminal tails. Acetylation of histone 3 at lysine residue 9 (H3K9) is one of the most common chromatin modifications associated with active gene expression, and therefore, this was analyzed by chromatin immunoprecipitation (ChIP) using antibodies specific for acetylated histone H3K9 in naïve, day 5 and day 8 effector P14 CD8 T cells. This chromatin immunoprecipitation with primers designed with in the *Il7ra* promoter from −294 to −59 bp revealed that the *Il7ra* promoter (−320 to −41 bp upstream of the translational start site) was hypoacetylated in day 5 effector cells than in naïve T cells (Fig. 1D). Interestingly, acetylation of the promoter region was regained, and perhaps somewhat increased, in day 8 effector CD8 T cells. This result was primarily due to the increased acetylation preferentially found in the IL-7Rα^{hi} effector CD8 T cell subset, which had ~4 fold greater acetylated H3K9 than did the IL-7Rα^{lo} subset (Fig. 1D). Furthermore, hypoacetylation of *Il7ra* promoter correlated with the recruitment of HDAC1 to the promoter, which was maximal at day 5 pi and remained elevated at day 8 pi, albeit at a level significantly lower than that observed at day 5 (Fig. 1E). Lastly, to verify whether *Il7ra* repression could be mediated through HDAC1 recruitment, via deacetylation of *Il7ra* locus, naive P14 CD8 T cells were stimulated with their cognate peptide ($GP_{33–41}$) in presence or absence of Trichostatin A (TSA), an HDAC inhibitor (33), and analyzed for surface IL-7Rα expression 24 hrs later. This experiment showed that TSA inhibited peptide-induced *Il7ra* repression of activated ($CD69^+$) CD8 T cells, confirming the involvement of HDACs in *Il7ra* repression (Fig. 1F). In summary, these data showed that CD8 T cells repress IL-7Rα as they expand and differentiate into effector CD8 T cells, and this is associated with recruitment of HDAC1 and histone deacetylation of the promoter region.

Il7ra downregulation is accompanied with the loss of GABPα binding in vivo

Expression of GABPα is necessary for IL-7Rα expression in thymocytes, but it is unclear if GABPα regulates IL-7Rα expression in effector and memory CD8 T cells. Therefore, we examined whether alterations in GABPα binding to the *Il7ra* promoter were associated with IL-7Rα mRNA downregulation in the effector CD8 T cells at days 5 and 8 pi. LCMV-specific P14 CD8 T cells were purified and the amount of GABPα bound to the *Il7ra* promoter was measured using ChIP with anti-GABPα antibodies. There was no significant difference in GABPα binding at day 5 pi as compared to naïve CD8 T cells (Fig. 2A), but surprisingly, the amount of GABPα bound to the promoter was ~2–3 fold higher in day 8 effector CD8 T cells compared to naïve or day 5 effector cells. Further analysis showed that it was primarily the subset of IL-7Rα^{hi} effector CD8 T cells at day 8 pi that contained increased GABPα promoter binding. Strikingly, even more GABPα was associated with the *Il7ra* promoter in memory CD8 T cells isolated 40–60 days pi. Together with the data in Figure 1, these results suggest that the occupancy of GABPα on the *Il7ra* promoter was important for IL-7Rα expression in MPECs and correlated with increased histone acetylation (Fig. 1D). Moreover, this analysis showed that even though naïve and IL-7Rα^{hi} effector and memory CD8 T cells express similar

amounts of IL-7Rα protein and mRNA, the naïve CD8 T cells achieve IL-7Rα expression with relatively less histone acetylation and GABPα binding. This may suggest that IL-7Rα^{hi} effector and memory CD8 T cells require additional post-transcriptional regulatory mechanisms, or relatively higher levels of histone acetylation and GABP binding in order to achieve similar IL-7Rα expression as naïve T cells.

GABPα is necessary for increased IL-7Rα expression in MPECs

To directly test the requirement of GABPα for increased IL-7Rα expression in IL-7Rα^{hi} MPECs, we used shRNAi to knock-down GABPα in LCMV-specific P14 effector CD8 T cells, similar to that done previously (29). Naïve P14 CD8 T cells were activated and 24hrs later transduced with either a control retrovirus (RV) or one that express sh*Gapb*α. The RV transduced cells could be identified by GFP expression. First, the efficiency of GABPα knock-down was verified by sorting on day 8 GFP+ P14 effector CD8 T cells and Western blotting for GABPα; this showed very efficient knockdown because the GFP+ CD8 T cells expressing sh*Gabp*α contained little to no detectable GABPα protein (Fig. 2B). Next, the RV transduced P14 CD8 T cells were adoptively transferred into LCMV infected wild type (WT) mice, and then a week later the donor GFP+ cells were examined for IL-7Rα expression. As expected, ~15–25% of the day 8 GFP+ P14 CD8 T cells transduced with control RV were IL-7Rα^{hi} (Fig. 2C, left plot). In contrast, the frequency of IL-7Rα^{hi} effector cells was substantially reduced in the GFP+ P14 CD8 T cells transduced with shGABPα RV (Fig 2C, right plot). These results demonstrated that the increased binding of GABPα to the IL-7Rα promoter in MPECs is functionally required for their ability to express IL-7Rα.

Gfi-1 regulates formation of IL7Rα^{lo} effector CD8 T cells

Next, we turned our attention to the transcription factor Gfi-1 because it has been shown to negatively regulate IL-7Rα expression in response to IL-7 signaling (23). However, it is not known if Gfi-1 functions in effector CD8 T cell differentiation during infection to repress IL-7Rα. To explore this possibility, we analyzed the expression of Gfi-1 mRNA in naïve and effector CD8 T cells and found that, as previously reported (34,35), Gfi-1 was not expressed in naïve CD8 T cells. Early after T cell activation Gfi-1 mRNA was induced and maintained at relatively high-levels throughout effector cell expansion and memory cell formation. However, there was no significant difference in Gfi-1 mRNA levels in IL-7Rα^{hi} and IL-7Rα^{lo} effector CD8 T cells at day 8 pi (data not shown). Thus, Gfi-1 and IL-7Rα expression do not appear to be inversely correlated as one may have predicted.

To determine if Gfi-1 bound to *Il7ra* locus during effector CD8 T cell differentiation we performed ChIP using anti-Gfi-1 antibodies (that do not cross-react with Gfi-1b) and analyzed binding of Gfi-1 within intron 2 of IL-7Rα as demonstrated previously (23) (see schematic in Fig. 1A). The level of Gfi-1 binding to *Il7ra* was nearly below detection in naïve *Gfi1*$^{+/+}$ CD8 T cells and *Gfi1*$^{-/-}$ effector CD8 T cells, confirming both the qRT-PCR data in naïve T cells and the specificity of the anti-Gfi-1 antibody used in these experiments. But, by day 5 pi the amount of Gfi-1 bound to *Il7ra* locus increased considerably (~15 fold increase; Fig. 3A). This correlated with the greatest repression of *Il7ra* transcription and HDAC1 recruitment (Fig.1). Surprisingly, Gfi1 binding to *Il7ra* was lower in day 8 effector CD8 T cells even though IL-7Rα expression was significantly repressed in more than 75% of the cells at this time. However, when day 5 and day 8 IL7Rα^{hi} and IL7Rα^{lo} CD8 T cells were separated and analyzed directly, Gfi-1 was preferentially bound to the *Il7ra* gene in the IL-7Rα^{lo} effector cells, demonstrating a strong correlation between Gfi-1 binding and IL-7Rα repression in virus-specific effector CD8 T cells (Fig. 3A). These data suggested that Gfi-1 is predominantly recruited to the *Il7ra* locus in effector CD8 T cells that will maintain stable repression of *Il7ra* and subsequently decline in number following infection.

To determine if the Gfi-1 binding to IL-7Rα was functionally relevant in effector CD8 T cells, the *Gfi1*$^{-/-}$ mice were crossed to the P14 TCR tg strain to obtain *Gfi1*$^{-/-}$ P14 CD8 T cells, and then small numbers of *Gfi1*$^{+/+}$ or *Gfi1*$^{-/-}$ P14 CD8 T cells were adoptively transferred into WT mice that were subsequently infected with LCMV. The levels of IL-7Rα protein and mRNA were examined on the donor cells at various times pi (Fig. 3B). Both naïve *Gfi1*$^{-/-}$ and *Gfi1*$^{+/+}$ P14 CD8 T cells expressed similar amounts of IL-7Rα at the protein and mRNA (Fig. 3B, left plots). At day 5 pi, IL-7Rα expression was reduced in LCMV-specific *Gfi1*$^{-/-}$ cells compared to naïve T cells, but not as profound as the reduction seen in *Gfi1*$^{+/+}$ CD8 T cells (Fig. 3B, middle plots). By day 8 pi, a small subset (~15–25%) of IL-7Rα^{hi} effector CD8 T cells formed in the *Gfi1*$^{+/+}$ population as expected. In contrast, a substantially larger percentage (~70–80%) of IL-7Rα^{hi} effector CD8 T cells formed in *Gfi1*$^{-/-}$ effector CD8 T cells (Fig. 3B, right plots). The mean fluorescence intensity of IL-7Rα in the effector CD8 T cells is shown (Fig. 3C, upper line graph). Accordingly, the expression of IL-7Rα mRNA followed a similar trend and was slightly elevated in day 5 *Gfi1*$^{-/-}$ effector CD8 T cells compared to *Gfi1*$^{+/+}$ cells, but the day 8 *Gfi1*$^{-/-}$ effector CD8 T cells contained significantly more IL-7Rα mRNA (Fig. 3C, lower graph).

To determine if the increased IL-7Rα expression in day 8 *Gfi1*$^{-/-}$ effector CD8 T cells correlated with decreased HDAC1 binding and/or increased histone acetylation, we performed ChIP assays in *Gfi1*$^{+/+}$ and *Gfi1*$^{-/-}$ P14 CD8 T cells. This analysis showed that significantly less (~7 fold) HDAC1 was bound to *Il7ra* in *Gfi1*$^{-/-}$ vs. *Gfi1*$^{+/+}$ day 8 LCMV-specific CD8 T cells (Fig. 3D), correlating with the increased IL-7Rα expression in day 8 Gfi-1$^{-/-}$ CD8 T cells. Moreover, the abundance of acetylated H3K9 on the IL-7Rα promoter in *Gfi1*$^{-/-}$ P14 effector CD8 T cells was nearly twice that of the *Gfi1*$^{+/+}$ cells (Fig. 3E). Together, these observations suggested that Gfi-1 was not absolutely required for the early IL-7Rα repression that occurs when viral infection was present (~day 5 pi), but may be needed subsequently to maintain stable IL-7Rα repression as antigenic stimulation declines by preserving HDAC1 recruitment and hypoacetylation of the promoter in the majority of terminally-differentiated effector cells.

Gfi-1 is required for stable repression of Il7ra in late effector CD8 T cells

The analysis above suggested that Gfi-1 was not sufficient for TCR driven repression of IL-7Rα (day 5 pi), but was needed for stable silencing of the locus in the absence of antigenic signaling (day 8 pi and later). To examine this point more closely, naïve *Gfi1*$^{+/+}$ or *Gfi1*$^{-/-}$ P14 CD8 T cells were stimulated with peptide *in vitro* for 12–24 hrs. As previously shown (13, 25), IL-7Rα was rapidly downregulated in *Gfi1*$^{+/+}$ CD8 T cells and Gfi-1 was recruited to the *Il7ra* locus in these early activated CD8 T cells (Fig. 4A and data not shown). Interestingly, the *Gfi1*$^{-/-}$ CD8 T cells exhibited a similar loss of IL-7Rα in response to TCR activation (Fig. 4A). Together, these findings suggested that even though Gfi-1 was recruited to *Il7ra* in activated T cells, a Gfi-1-independent mechanism largely operates downstream of TCR signaling early on to repress *Il7r*. This helps to explain the nearly normal decrease in IL-7Rα observed in *Gfi1*$^{-/-}$ LCMV-specific effector CD8 T cells at day 5 pi when virus was still prevalent (Fig. 3B, C).

Next, we tested if the abnormally swift transition from IL-7Rα^{lo} to IL-7Rα^{hi} found in *Gfi1*$^{-/-}$ effector CD8 T cells between days 5–8 pi was due to direct conversion (i.e., the inability to sustain *Il7ra* silencing in late effector *Gfi1*$^{-/-}$ CD8 T cells) or to the preferential death of IL-7Rα^{lo} *Gfi1*$^{-/-}$ cells. At day 5 pi, *Gfi1*$^{+/+}$ and *Gfi1*$^{-/-}$ P14 CD8 T cells (which were congenically marked Thy1.1^{+}) were sorted by FACS based on IL-7Rα^{lo} expression and then equal numbers were adoptively transferred separately into congenic (Thy1.2) recipients that were infected 5 days previously (Fig. 4B). At day 8 pi (3 days later), the IL-7Rα^{lo} donor cells were examined for IL-7Rα expression. As expected, the majority of the *Gfi1*$^{+/+}$ effector CD8 T cells maintained repression of IL-7Rα over this time. In contrast, most of the donor

Gfi1$^{-/-}$ effector CD8 T cells could not sustain IL-7Rα transcriptional repression and ~60% of the cells converted to an IL-7Rα^{hi} state within three days (Fig. 4B). These data indicated that at least two mechanisms of IL-7Rα repression exist in effector CD8 T cells: one form being driven by TCR signaling that does not require Gfi-1, and another that stably maintains *Il7ra* repression in the absence of antigenic signals and requires Gfi-1.

Gfi-1 over expression represses Il7ra by reducing GABPα binding to Il7ra

To further investigate the role of Gfi-1 in IL-7Rα expression in effector CD8 T cells, Gfi-1 was over expressed in LCMV-specific CD8 T cells using RV transduction. P14 CD8 T cells were activated and transduced with control or Gfi-1 RV and either transferred into LCMV infected or naïve recipients; the RV transduced cells were marked by GFP expression. This experiment revealed that over expression of Gfi-1 inhibited the development of a subset of IL-7Rα^{hi} effector cells during infection (Fig. 5A, right plot). Moreover, when P14 CD8 T cells were briefly stimulated with peptide for 24hrs *in vitro* (under low inflammatory conditions) IL-7Rα repression was transient for the first 24–36 hrs and was then regained as the activated CD8 T cells continued to clonally expand (Fig. 4A and data not shown). Using this system, Gfi-1 RV over expression was sufficient to repress IL-7Rα in nearly all of the briefly stimulated and expanded CD8 T cells (Fig. 5B, right plot).

To assess the effects of Gfi-1 over expression on histone acetylation of the *Il7ra* promoter, GFP+ (Gfi-1 RV) and GFP- (no RV) P14 CD8 T cells were sorted and the amount of acetylated H3K9 was measured using ChIP assays. This analysis showed that Gfi-1 over expression resulted in profound hypoacetylation of the *Il7ra* promoter (Fig. 5C). These data in combination with those shown in Fig. 3 demonstrate that Gfi-1 is necessary and sufficient (when over expressed) to maintain repression of *Il7rα*, perhaps via histone H3 deacetylation, during effector CD8 T cell differentiation.

The above data suggested that GABPα and Gfi-1 have opposing effects on *Il7rα* transcription; GABPα binding enhances promoter acetylation and transcription whereas Gfi-1 promotes HDAC binding, promoter deacetylation and transcriptional silencing. A possibility could be that over expression of Gfi-1 inhibits binding of the transcriptional activator GABPα to the *Il7ra* promoter. To examine this question, we measured the amounts of GABPα bound to *Il7ra* promoter using ChIP when Gfi-1 was absent (i.e., *Gfi1*$^{-/-}$ effector CD8 T cells) or over expressed by RV transduction. This showed that the amount of GABPα bound to the *Il7ra* promoter in day 8 *Gfi1*$^{-/-}$ effector CD8 T cells was ~1.5–2 fold greater than in *Gfi1*$^{+/+}$ cells (Fig. 5D). This result suggested that GABPα function is important for the increased IL-7Rα expression in *Gfi1*$^{-/-}$ effector CD8 T cells. Next, the day 8 effector P14 CD8 T cells over expressing Gfi-1 were sorted and GFP+ (Gfi-1 RV) and GFP- (no RV) were compared for GABPα expression and binding. qRT-PCR confirmed that there was no difference in GABPα mRNA levels (data not shown) in GFP+ and GFP- P14 CD8 T cells, showing that Gfi-1 over expression did not affect GABPα expression (data not shown). However, ChIP analysis of GABPα revealed that GFP+ P14 CD8 T cells had ~20.5 fold less GABPα bound to their *Il7ra* locus compared to GFP- P14 CD8 T cells (Fig. 5E), indicating that over expression of Gfi-1 reduces GABPα binding to *Il7ra* promoter.

Discussion

This study was designed to understand the molecular mechanism(s) that control IL-7Rα expression in CD8 T cell with expansion and differentiation of IL-7Rα^{hi} MPECs and IL-7Rα^{lo} SLECs in response to an acute viral infection. We found that IL-7Rα expression is regulated in these two effector cell populations by a balance between the transcriptional repressor Gfi-1 and the transcriptional activator GABPα. Here, our results suggest that the *Il7ra* locus exists in multiple states as naïve CD8 T cells differentiate into MPECs and SLECs.

In the first state, in resting naïve CD8 T cells, Gfi-1 is not expressed, *Il7ra* is transcriptionally active and the promoter is acetylated with moderate levels GABPα binding. Upon activation, TCR signaling causes rapid downregulation of IL-7Rα expression and this is correlated with hypoacetylation of the promoter region and recruitment of HDAC1. Gfi-1 is induced early in activated T cells and recruited to the *Il7ra* locus (data not shown), but does not appear essential for early TCR-dependent *Il7ra* repression at this stage. As the viral infection is resolved and effector CD8 T cell expansion peaks around ~ day 8 post infection, the MPECs and SLECs became easier to discern based on high and low levels of IL-7Rα expression respectively. The majority of the effector CD8 T cells express little to no IL-7Rα and are considered terminally differentiated because they have lost the ability to become long-lived memory CD8 T cells. The reduced IL-7Rα expression in this subset was associated with increased HDAC1 recruitment and decreased GABPα binding and histone acetylation of the promoter. Gfi-1 preferentially bound to the *Il7ra* locus and was required for stable *Il7ra* repression in these cells. When Gfi-1 was over expressed the occupancy of GABPα and promoter acetylation was reduced even further, suggesting that in addition to recruiting HDACs, Gfi-1 also functions to repress *Il7ra* by inhibiting recruitment of a requisite transcriptional activator GABPα.

The *Il7ra* locus existed in the opposite state in the smaller population of IL-7Rα^{hi} MPECs where GABPα binding to and histone acetylation of the promoter was abundant, and Gfi-1 binding to intron 2 was significantly decreased. Furthermore, GABPα was functionally required to sustain IL-7Rα expression in these IL-7Rα^{hi} MPECs. Therefore, since IL-7Rα expression is functionally required for generation of a pool of memory CD8 T cells, GABPα is likely required for memory CD8 T cell development and maintenance.

These data offer a model for how the “on” or “off” state of *Il7ra* is mediated by the binding of opposing transcriptional activators and repressors and concurrent epigenetic remodeling of the *Il7ra* promoter. Interestingly, the primary determinant of *Il7ra* transcriptional regulation was not necessarily the differential expression of GABPα or Gfi-1 in IL-7Rα^{hi} or IL-7Rα^{lo} effector CD8 T cells, but rather it was the preferential recruitment of GABPα or Gfi-1 to the *Il7ra* locus in these respective T cell subsets. How is this reciprocal binding pattern of Gfi-1 and GABPα set-up and maintained in the effector CD8 T cells? GABPα is a target of phosphorylation by certain kinases such as ERK and JNK (36) (37), and perhaps, differential activity of these kinases in IL-7Rα^{hi} and IL-7Rα^{lo} effector CD8 T cells contributes to *Il7ra* gene regulation. Another possibility is that other signals (aside from TCR activation) stabilize Gfi-1 binding in the majority of effector CD8 T cells and this directly impairs GABPα docking to the promoter. Evidence for this idea is provided by prior work that showed that Gfi-1 can antagonize the transcriptional activation of ets-family members, ETS-1 and PU.1, by direct binding (38,39). Another possibility is that the transcription factor T-bet (*Tbx21*) cooperates with Gfi-1 to antagonize GABPα activity in effector CD8 T cells. Our recent work has shown that certain inflammatory signals during infection, such as IL-12, directly increase T-bet expression in effector CD8 T cells and this induces development of IL-7Rα^{lo} SLECs (20). Like Gfi-1 RV over expression, T-bet RV over expression represses IL-7Rα expression in effector CD8 T cells (20,40). Therefore, it is possible that increased T-bet stabilizes Gfi-1 recruitment to the *Il7ra* locus in effector CD8 T cells that were exposed to high levels of IL-12, but this question remains to be tested.

As might be expected for a protein whose expression is critical to naïve and memory T cell generation and maintenance, IL-7Rα expression appears to be tightly regulated by multiple signals and mechanisms. TCR activation leads to rapid IL-7Rα downregulation, but this TCR-directed repression is transient and the mechanism by which this occurs is not known. We show here that TCR-dependent inhibition of IL-7Rα expression is largely independent of Gfi-1 both *in vitro* and *in vivo* (when viral antigens are present). Moreover, this process is also T-bet independent (data not shown). However, we found that Gfi-1 was necessary to sustain a stable

repressed state in late effector CD8 T cells (~day 7 onwards) when viral infection and antigenic stimulation was ceasing. Thus, at least two mechanisms exist, which occur over two phases during infection, that inhibit IL-7Rα expression in activated CD8 T cells during viral infection. One is transient repression mediated by TCR signaling, and the other is stable repression that occurs primarily in terminally differentiated effector CD8 T cells and requires Gfi-1.

Our analysis showed that *Il7ra* repression in activated CD8 T cells was also associated with characteristic epigenetic chromatin modifications, namely histone (H3K9) deacetylation via HDAC1. A previous report showed that increased CpG DNA methylation in the promoter of IL-7Rα^{lo} CD8 T cells was likely important for maintenance of IL-7Rα^{lo} state in resting effector memory T cells (T_{EM}) (41). However, DNA methylation did not seem to be involved in the rapid TCR- or IL-7-directed repression of *Il7r*α (41). Thus, the epigenetic control of *Il7ra* expression appears to involve multiple layers to ensure tight, yet long term, regulation of either high and low IL-7Rα expression levels in T cells.

Some γ_c chain cytokines and steroids have also been found to regulate IL-7Rα expression (13,23,24,26). IL-7 and several other γ_c chain cytokines induce rapid IL-7Rα downregulation that is transient in nature because the T cells quickly regain IL-7Rα expression following removal from high dose cytokine exposure (23,24). It was hypothesized that this temporary form of IL-7Rα down regulation was an altruistic way by which T cells could decrease competition for the limiting amounts of IL-7 in the periphery (23). This mode of repression contrasts to the more “permanent” form of IL-7Rα repression found during effector CD8 T cell development (12,20). However, in CD8 T cells, but not CD4 T cells, this cytokine-dependent silencing is Gfi-1 dependent (23). Glucocorticoids provide another signal that upregulate IL-7Rα expression via binding of the glucocorticoid receptor (GR) to a GR binding site in a small evolutionary conserved region (ECR) ~3.6kb upstream of the *Il7ra* promoter (13,26). Using rVista 2.0 analysis (42), multiple other TF binding sites (such as Foxo1/3/4, NFκβ, Gata6, Pax3 and Stat1/4) are predicted in this region and serve as additional candidates to regulate IL-7Rα expression. In addition to this ECR, at least two others can be found using this analysis in the 6 kb upstream of the *Il7ra* promoter, but the relevance of these ECRs and the potential TFs that bind to these regions remains to be determined.

Our data shed light on the biochemical processes by which effector CD8 T cells gain or lose memory CD8 T cell potential. Currently, very low amounts of surface IL-7Rα is one of the best markers for effector or memory CD8 T cells that are terminally-differentiated and short-lived during infections (12,14,16). Therefore, understanding how the repression of IL-7Rα is initiated and sustained in anti-viral CD8 T cells is key to understanding how this differentiated state is acquired. In addition to serving as a marker, IL-7Rα downregulation was initially predicted to be the underlying cause of death of the majority of the effector CD8 T cells after infection since IL-7 deprivation causes T cell apoptosis (1,7). However, our recent data and that of others have shown that the bulk of effector T cell contraction that occurs following infection is IL-7 independent. (21,43,44). When IL-7Rα expression was enforced on all virus-specific effector CD8 T cells using an *Il7ra* transgene, this did not rescue the preferential death of the naturally arising IL-7Rα^{lo} effector CD8 T cell subset (21). Other work showed that this IL-7Rα^{lo} subset is mainly dependent on IL-15 for survival, however, IL-15 does not suffice to maintain the IL-7Rα^{lo} CD8 T cells long-term (20). In contrast, the IL-7Rα^{hi} MPECs functionally require IL-7Rα to persist and develop into long-lived memory CD8 T cells (12, 17,45). Therefore, elucidating that GABPα is required for expression of IL-7Rα in these cells is an important finding for memory CD8 T cell development.

Numerous target genes have been identified for both GABPα and Gfi-1 (30,46–48), and therefore, it is important to consider the additional effects, aside from abnormal IL-7Rα expression, that result in GABPα and Gfi-1-deficient effector CD8 T cells. Interestingly, in

addition to IL-7Rα, a few other genes such as IL-2, TNFα, and ELA2 are predicted to contain binding sites for both Gfi-1 and GABPα, and it is possible that these two factors play opposing roles on their gene expression (47,49–51). It is noteworthy that GABPα also controls the expression of several genes involved in mitochondrial function, cellular energy metabolism and cell cycle progression (30,52). It is possible that the coregulated expression of these genes with *Il7ra* (via GABPα)in MPECs is vital to their cellular fitness and ability to become long-lived memory CD8 T cells with a high proliferative potential.

A long-term goal is to elucidate the transcriptional network that controls effector and memory CD8 T cell differentiation and to identify key transcriptional regulators. Beginning with a gene vital to memory CD8 T cell development and survival, IL-7Rα, this work provides inroads into this extremely complex problem. In the future it will be of value to find other genes that are co-regulated with IL-7Rα, to identify their transcriptional regulators, and to identify if these represent a cassette of genes critical to memory CD8 T cell formation.

Materials and Methods

Mice and infections

C57BL/6 (B6) mice were purchased from National Cancer Institute (Fredrick, MD) and Jackson Laboratories (Bar Harbor, ME). Thy1.1^{+} P14 TCR tg mice have been described previously (53). *Gfi1*$^{-/-}$ mice were generously provided by Dr. Stuart Orkin (Children's hospital, Boston, MA) (54). For experiments involving P14 tg CD8 T cells, ~1×10^{4} *Gfi1*$^{+/+}$ or *Gfi1*$^{-/-}$ P14+ CD8 T cells were transferred to make "P14 chimeric mice". Mice were infected i.p. with 2×10^{5} PFU of LCMV-Armstrong strain (53).

Cell isolations and separations

Populations of Thy1.1+ CD8 P14 T cells were purified by magnetically depleting non-CD8 T cells using antibodies to CD4 (GK1.5), B220 (TIB164 and H-2^{b} (TIB120) obtained from K. Bottomly (Yale University, New Haven, CT), followed by anti-rat IgG and mouse-IgM magnetic beads (Polysciences Inc, Warrington, PA) and sorted using FACSAria (BD) or MoFlo (DakoCytomation) cell sorters. Equal numbers of sorted cells were transferred i.v. into B6 recipient mice.

Retroviral (RV) constructs and transduction

P14 Tg mice were directly infected with 2×10^{6} pfu LCMV-Armstrong i.v. and 1 day later, P14 Tg splenocytes were spin transduced for 90 min at 37°C with fresh viral supernatants from 293T cells (transfected 48 hours prior with Eco helper and either MigR1 GFP control, MigR1-*Gfi-1*-GFP (55), MSCV-Mir-GFP (MicroRNA cassette containing Xho1 and EcoR1 sites from the LMP vector (Open Biosystems, Huntsville, AL) was cloned into the MigR1 GFP vector) or MSCV-Mir-sh*Gabpα*–GFP in the presence of 8ug/ml of polybrene and 10ug/ml of IL-2 and then immediately transferred i.v. to C57BL/6 mice that were subsequently infected with 2×10^{5} PFU LCMV. For experiments involving transfer into naïve mice, ~10×106 P14 CD8 T cells were cultured for 24 hours with 100ng/ml $GP_{33–41}$ peptide and spin transduced as described above to avoid transfer of LCMV into naïve mice. Mice containing RV-transduced P14 CD8 T cells were sacrificed at day 5 or 8 post transduction and GFP+ P14 CD8 T cells were analyzed by flow cytometry or sorted using FACSAria (BD) or MoFlo (DakoCytomation) cell sorters.

Real-time PCR analyses

For real time analysis ~2×10^{5} naïve P14 CD8 T cells, day 5–8 P14 effector CD8 T cells, day 8 IL-7Rα^{hi} and IL-7Rα^{lo} cells, were sorted by FACS. Total RNA was extracted by Trizol

method using the manufacturers instructions and cDNA was synthesized using SSRTII (Invitrogen). Real-time PCR analysis was performed as previously described (56). Primers for *IL-7Rα*: 5'-GCAGACGCGGACCATCACTC-3' and 5'- ATTTTTGCAAGTTAAATTCT-3'; *Gfi-1* 5' CTCATTCCTGGTCAAGAGC-3' and 5'-CATGCATAGGGCTTGAAA-3' Due to the extensive homology between *Gfi1* and its homologue *Gfi1b*, real time PCR primers were chosen in the non homologous regions of the *Gfi1* cds: *L9*: 5'-TGAAGAAATCTGTGGGTCG-3' and 5 ' - GCACTACGGACATAGGAACTC-3'. *L9* is a ribosomal protein gene that is expressed at a relatively constant level in naïve, effector, and memory CD8 T cells and served as an internal reference. All PCRs used an annealing temp 55°C. Relative fold differences were calculated as described (57).

Western blotting

Protein lysates from 1×10^6 sorted naïve cells ($CD8^+$ $CD44^{lo}$) or day 5 and day 8 sorted total effector CD8 T cells were and resolved by SDS-PAGE. Antibodies to GABPα (29), was used for Western blotting at 1:1000 dilution. The blots were stripped and reprobed with Grp94 (Cell Signal, Danvers, MA) at a dilution of 1:5000 as normalization control.

Chromatin Immunoprecipitation

ChIP assays were performed with standard procedures (Upstate Biotech, Temecular, VA, USA). Briefly, DNA was crosslinked with formaldehyde (1% final concentration) and chromatin was sheared by sonication until an average length of DNA ladder of 500 bp increments was obtained. Salmon sperm DNA/protein agarose (Upstate Biotech, Temecula, VA, USA) was used for preclearing and immunoprecipitation. Antibodies against acetyl H3K9 and HDAC1 (Upstate Biotech, Temecula, VA), GABPα (29), and Gfi-1 (gift from Dr. H Bellen, raised against the SNAG domain that is non-homologous regions between Gfi-1 and Gfi-1B and is not reactive in $Gfi1^{-/-}$ mice) (58) were used for precipitating the protein-DNA concentration. Precipitated protein–DNA complexes were washed in low-salt buffer (20 mM Tris-HCl pH 8.1, 2 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate and 1% Triton X-100), and with TE (10 mM Tris-HCl pH8.1, 1 mM EDTA) buffer twice. The precipitated protein–DNA complexes were eluted and crosslinks were reversed with NaCl (200mM final concentration) incubated at 65°C overnight. After treatment with RNase A and proteinase K, DNA was extracted with phenol/chloroform and precipitated with ethanol. Purified DNA was then subjected to 40 cycles of PCR amplification. Primers spanned −294 to −59 region within the *Il7ra* promoter and the reported binding site for Gfi-1 in intron 2. The primer sequences for *Il7ra* promoter forward 5' GCAGTTAAGTTCAGGAGCTTCAGG 3' and reverse 5' GAAGCACGGTTGTATGTGCAAGTG 3'; *Il7ra* Intron 2 forward 5'CCAGACTATCTAGTCAATGG 3' and reverse 5' TTCAAGTCACCAGAGATAAT 3'. DNA concentration was calculated from a genomic DNA standard 8-fold dilution series and in most cases fold differences between samples were calculated by normalizing to naive CD8 T cells set at one or calculated as relative DNA concentration with respect to genomic DNA curve and the initial input DNA.

Statistical Analyses

Where indicated, p-values were determined using a two-tailed unpaired student's t-test. p-values <0.05 were considered significant. All graphs show averages of the mean ± s.e.m.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CLP	Common lymphoid progenitor
Pi3K	Phosphoinositide – 3 - kinase
AKT	Serine threonine protein kinase B (AKT mouse thymoma)
ERK	extracellular signal-regulated kinase
JNK	c-Jun N-terminal Kinase

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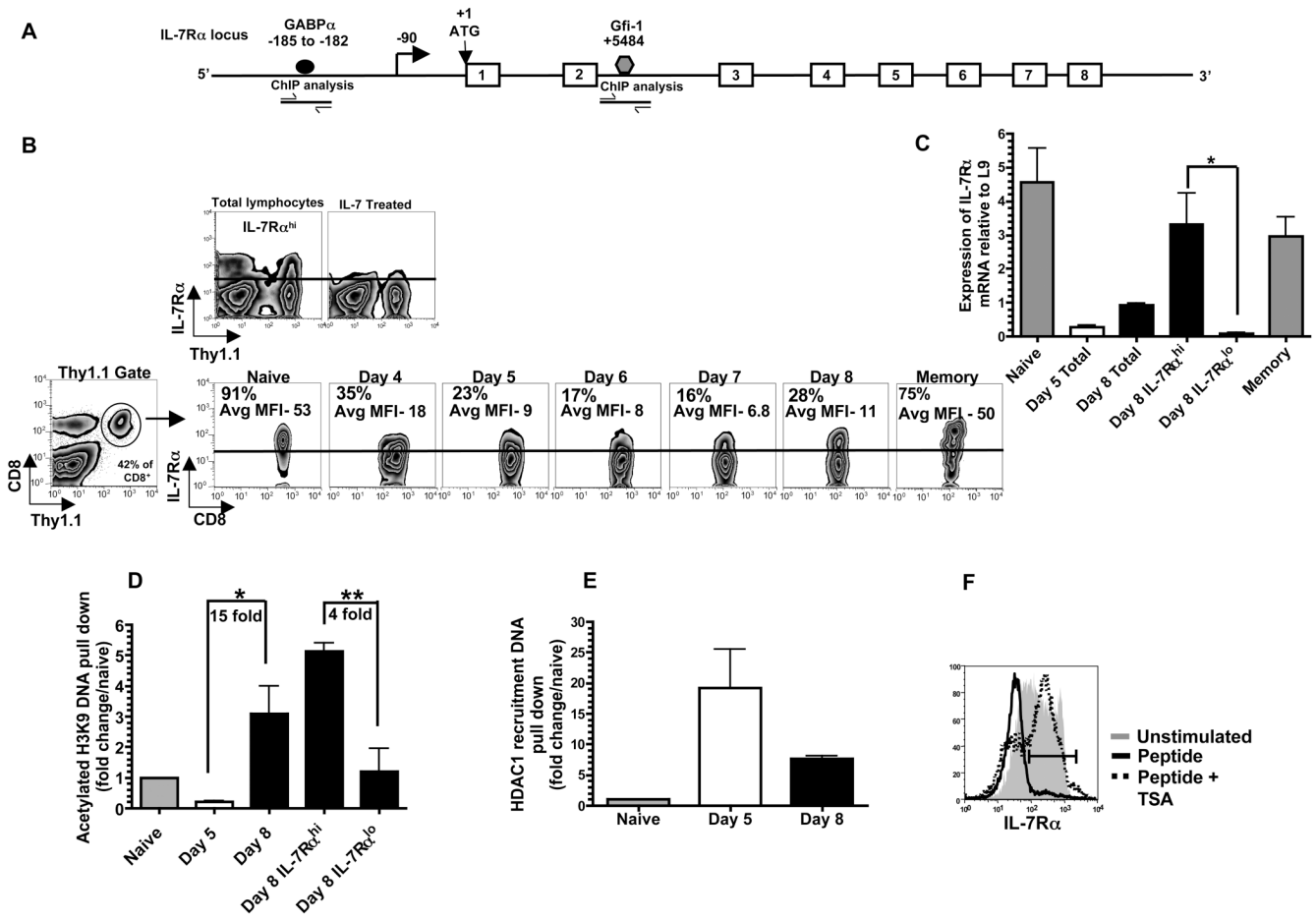

Figure 1. Transcriptional repression of il7rα in LCMV specific effector CD8 T cells is associated with histone deacetylation and HDAC1 recruitment

A. Map of *Il7rα*.*Il7ra* has eight exons shown as numbered rectangles. The transcriptional start site is +1, and GABPα binds at –185 bp and Gfi-1 binds at +5484 bp. The region of DNA analyzed for GABPα binding, promoter acetylation, HDAC1 recruitment and Gfi-1 binding by ChIP are denoted.

B–C. IL-7Rα is down regulated in CD8 T cells after LCMV infection. Naïve P14 CD8 T cells (Thy1.1$^+$) were adoptively transferred into wild type recipients (Thy1.2$^+$) to make "P14 chimeric mice" that were subsequently infected with LCMV. The amount of IL-7Rα protein and mRNA was measured in P14 CD8 T cells from naïve P14 mice or P14 chimeric mice infected 4–8 days previously using flow cytometry **(B)** and qRT-PCR **(C)**. In **(B)**, top row plots show gating criteria for IL-7Rα^{hi} and IL-7Rα^{lo} CD8 T cells, which were based on IL-7Rα expression of total lymphocytes because IL-7Rα^{hi} and IL-7Rα^{lo} cells are clearly visible (top row, left plot) and CD8 T cells treated with IL-7 because this leads to rapid IL-7Rα downregulation (top row, right plot). Bottom row plots show expression of IL-7Rα on Thy1.1$^+$ P14 CD8 T cells from naïve or infected animals. The percentage of IL-7Rα^{hi} cells and the average mean fluorescent intensity (MFI) of the total population is indicated in each plot. **(C)** Thy1.1+ P14 CD8 T cells were FACS sorted from naïve, infected (days 5 and 8 pi) or immune (day 50+ pi) mice. P14 CD8 T cells from day 8 p.i. were also divided into IL-7Rα^{hi} and IL-7Rα^{lo} subsets. IL-7Rα mRNA levels were measured from these cell populations using qRT-PCR. Bar graph shows IL-7Rα mRNA levels relative to L9 ribosomal

protein gene (a gene whose expression does not vary much with CD8 T cell activation). Data are mean ± SEM (n=3–6); *=p<0.05.

D–E. IL-7Rα repression after LCMV infection coincides with decreased acetylation and HDAC1 recruitment. Naïve, day 5, day 8 or IL-7Rα^{hi} and IL-7Rα^{lo} day 8 P14 effector CD8 T cells were purified using FACS and analyzed for acetylated H3K9 histones (**D**) or HDAC1 binding (**E**) on the *Il7ra* promoter using chromatin immunoprecipitation followed by qRT-PCR. The data in bar graphs were plotted as fold changes in acetylation or HDAC1 recruitment with respect to naïve T cells set at 1 fold. The graph shows mean ± SEM (n=3); *=p<0.05 and **=p<0.01.

F. IL-7Rα repression is TSA sensitive. CD8 T cells were stimulated over night with LCMV peptide $GP_{33–41}$ with (dashed line) or without (thick line) a non-specific HDAC inhibitor, trichostatin A. Histogram shows the expression of IL-7Rα in CD69+ activated CD8 T cells relative to unstimulated, naïve CD8 T cells (filled histogram).

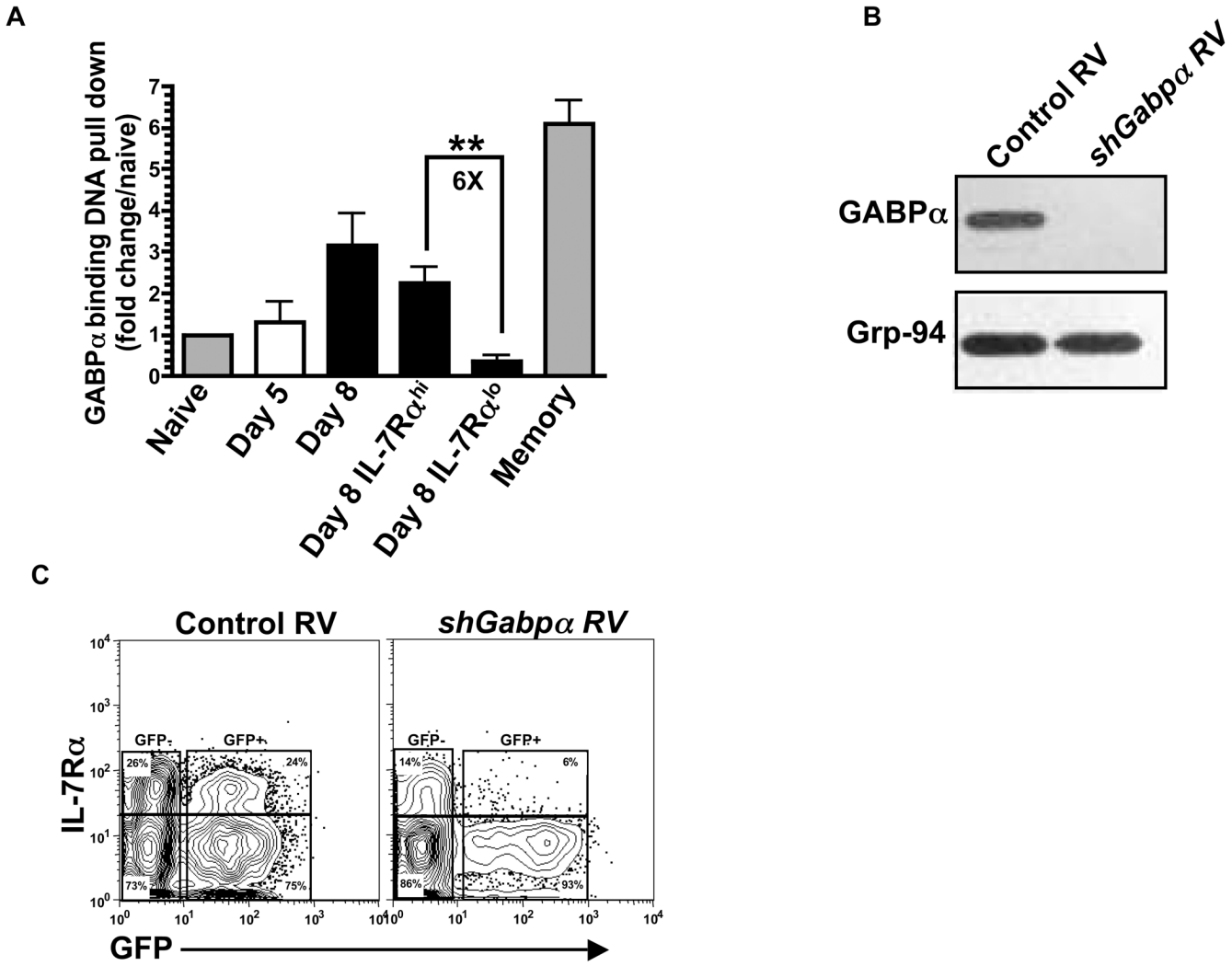

Figure 2. GABPα is necessary for IL-7Rα expression in LCMV-specific effector CD8 T cells

A. Binding of GABPα to *Il7ra* promoter correlates with IL-7Rα expression. Naïve, day 5, day 8 or IL-7Rα^{hi} and IL-7Rα^{lo} day 8 P14 effector CD8 T cells were purified using FACS and analyzed for GABPα binding on the *Il7ra* promoter using chromatin immunoprecipitation followed by qRT-PCR. Bar graph represents fold differences in GABPα pull down with naive CD8 T cells set at 1 fold. The data are mean ± SEM (n=6); **=p<0.01.

B–C. GABPα is necessary for the formation of IL-7Rα^{hi} effector CD8 T cells. Activated P14 CD8 T cells were transduced with RVs (marked by GFP+ expression) expressing sh*Gabp*α RNAi or empty control vector and subsequently transferred into LCMV infected recipients. Seven days pi GFP+ P14 CD8 T cells were analyzed for expression of GABPα (**B)** and IL-7Rα (**C**). In (**B**), the GFP+ P14 CD8 T cells were purified by FACS and protein lysates were analyzed for expression of GABPα and Grp-94 (protein loading control) by Western blotting. In (**C**), the GFP+ and GFP- P14 CD8 T cells were analyzed for surface IL-7Rα expression using flow cytometry. Note, specific reduction in the frequency of IL-7Rα^{hi} effector CD8 T cells in GFP+ P14 CD8 T cells transduced with sh*Gabp*α RNAi RV.

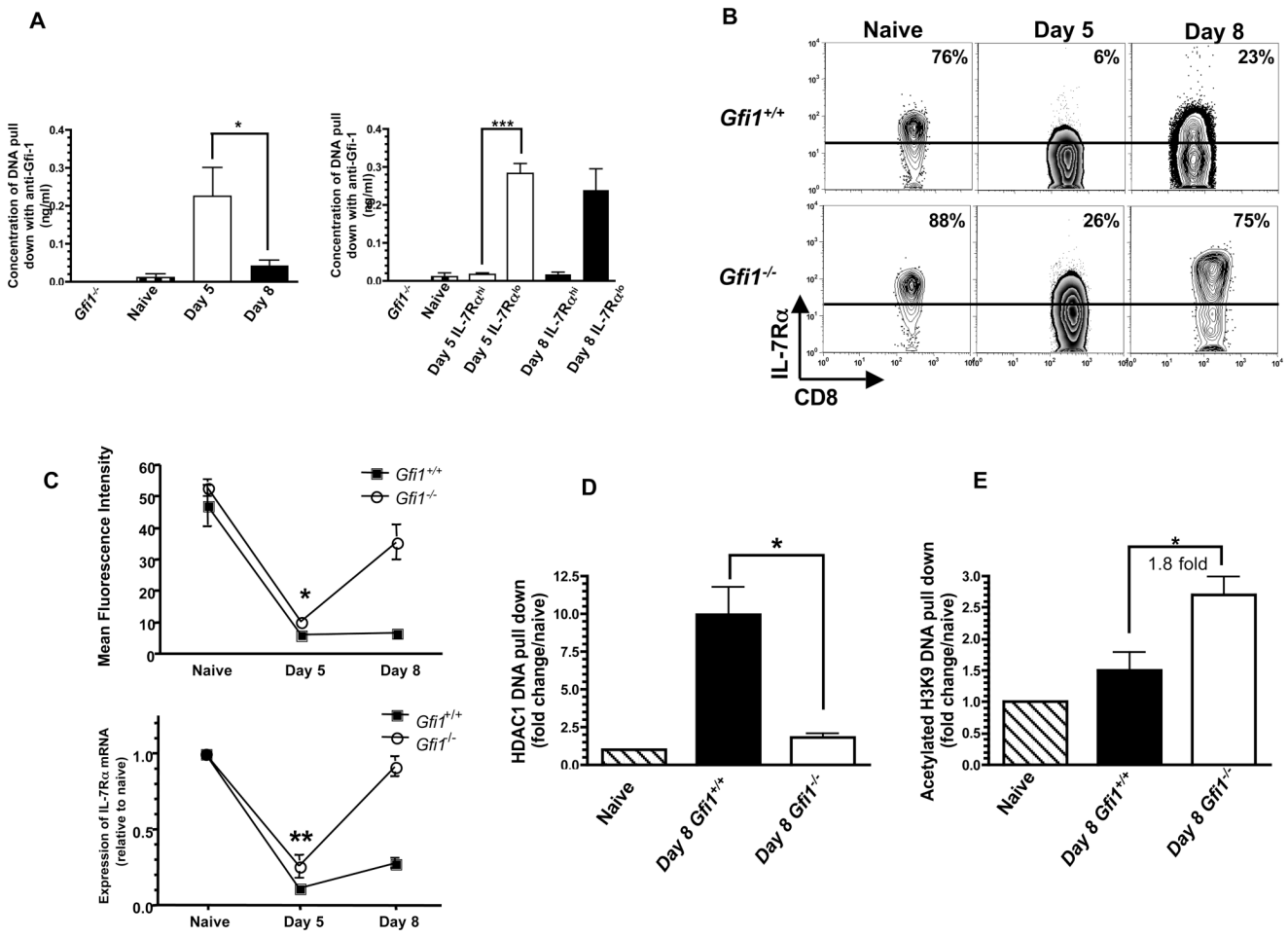

Figure 3. Gfi-1-mediated IL-7Rα repression in 'late' effector CD8 T cells is associated with decreased HDAC1 binding and increased promoter acetylation

A. Gfi-1 preferentially binds to IL-7Rα in IL-7Rα^{lo} effector CD8 T cells. Naïve, day 5, day 8 or IL-7Rα^{hi} and IL-7Rα^{lo} day 5 or day 8 P14 effector CD8 T cells were purified using FACS and analyzed for Gfi-1 binding on intron 2 using chromatin immunoprecipitation followed by qRT-PCR. *Gfi1*$^{-/-}$ CD8 T cells were used to correct against background signal. Bar graph shows the amount of DNA immunoprecipitated from each sample in ng/ml, based on a genomic standard. The graph in data are mean ± SEM (n=4); *=p<0.05.

B–C. Gfi-1 is required for IL-7Rα repression in 'late' effector CD8 T cells. P14 chimeric mice containing LCMV-specific *Gfi1*$^{+/+}$ and *Gfi1*$^{-/-}$ P14 CD8 T cells were infected with LCMV and analyzed for IL-7Rα expression by flow cytometry (**B** and **C, upper graph**) and qRT-PCR (**C, lower graph**) at day 5 and 8 pi. Naive *Gfi1*$^{+/+}$ and *Gfi1*$^{-/-}$ P14 CD8 T cells were also analyzed. (**B**) Dot plots are gated on P14 CD8 T cells and the percent IL-7Rα^{hi} CD8 T cells is indicated. (**C, upper graph**) The mean fluorescence intensity (MFI) of IL-7Rα expression on the *Gfi1*$^{+/+}$ (closed squares) and *Gfi1*$^{-/-}$ (open circles) populations of LCMV-specific P14 CD8 T cells are shown. Data are mean ± SEM (n=8); *=p<0.05 (**C, lower graph**) qRT-PCR analysis of IL-7Rα mRNA in *Gfi1*$^{+/+}$ (filled squares) and *Gfi1*$^{-/-}$ (open circles) effector P14 CD8 T cells purified by FACS. Each time point shows mean ± SEM (n=3); **=p<0.01.

D–E. Gfi1−/− effector CD8 T cells exhibit decreased HDAC1 binding and increased histone acetylation at the IL-7Rα promoter. Day 8 *Gfi1*$^{+/+}$ or *Gfi1*$^{-/-}$ P14 effector CD8 T

cells were purified using FACS and analyzed for HDAC1 binding (**D**) or acetylated H3K9 histones (**E**) or on the *Il7ra* promoter using chromatin immunoprecipitation followed by qRT-PCR. The data in bar graphs were plotted as fold changes with respect to naïve *Gfi1*$^{+/+}$ CD8 T cells set at 1 fold. Each time point shows mean ± SEM (n=3); *=p<0.05

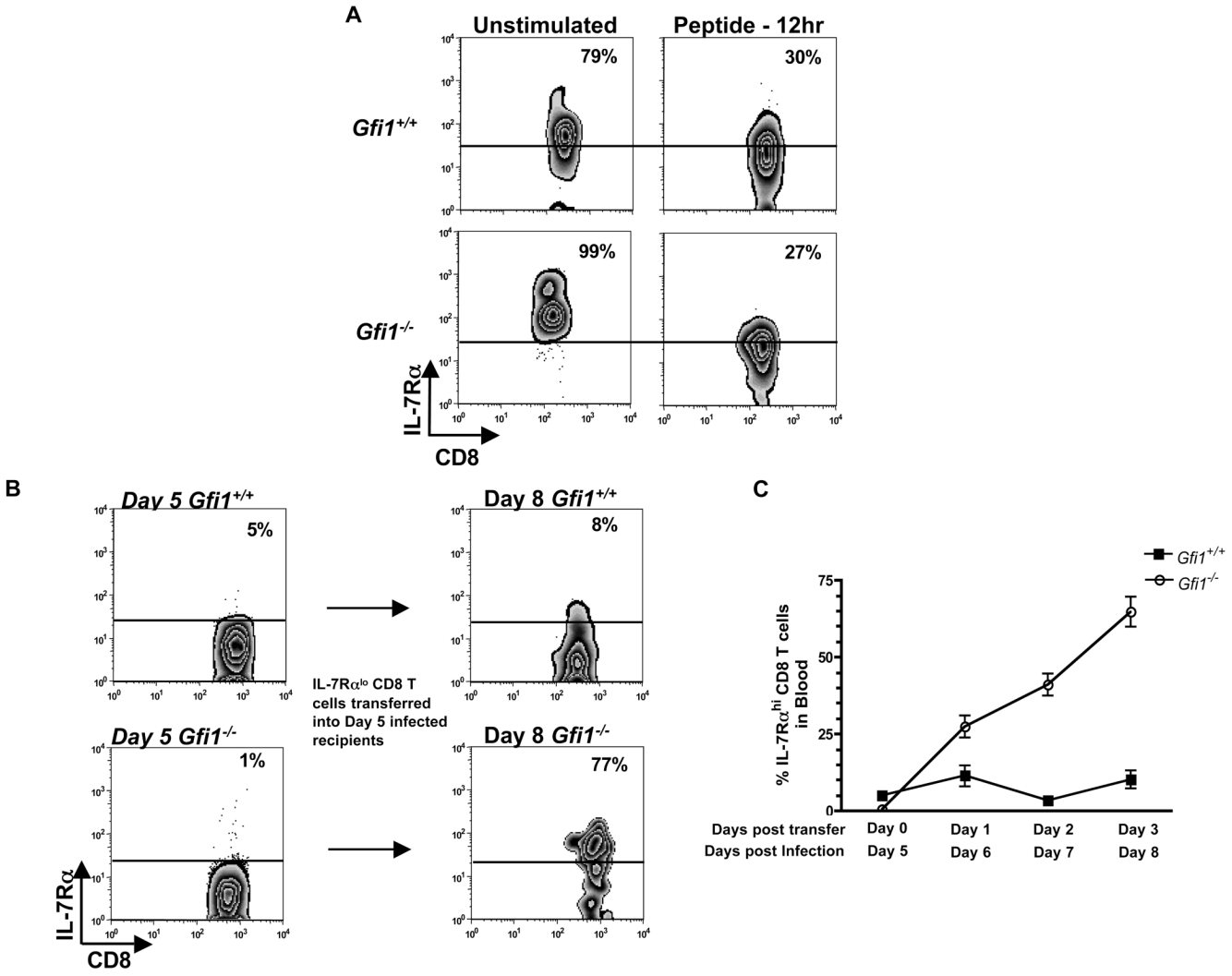

Figure 4. Gfi-1 is required for stable repression of IL-7Rα in effector CD8 T cells during infection

A. IL-7Rα repression following *in vitro* peptide stimulation is transient. *Gfi1*$^{+/+}$ (upper panel) and *Gfi1*$^{-/-}$ (lower panel) P14 CD8 T cells were stimulated with $GP_{33–41}$ peptide for 12–48 hrs and IL-7Rα expression was measured on activated (CD69^{hi}) CD8 T cells using flow cytometry. Contour plots are gated on P14 CD8 T cells and the percent IL-7Rα^{hi} cells is shown. Data in plots are representative of 4 independent experiments.

B–C. *Gfi1*$^{-/-}$ effector CD8 T cells cannot maintain stable repression of IL-7Rα as infection wanes. On day 5 pi, *Gfi1*$^{+/+}$ (upper panels) or *Gfi1*$^{-/-}$ (lower panels) P14 CD8 T cells were purified based on IL-7Rα^{lo} staining by FACS and transferred separately into day 5 LCMV infected recipients (left plots show sort purity). (**B**) Three days later (day 8 pi) donor P14 CD8 T cells in the spleen were analyzed for IL-7Rα expression by flow cytometry (right plots). Contour plots are gated on P14 CD8 T cells and the percent of IL-7Rα^{hi} cells is indicated. (**C**) Line graph shows percent IL-7Rα^{hi} *Gfi1*$^{+/+}$ (filled squares) and *Gfi1*$^{-/-}$ (open circles) donor effector P14 CD8 T cells in the blood each day post transfer. Data are mean ± SEM (n=4).

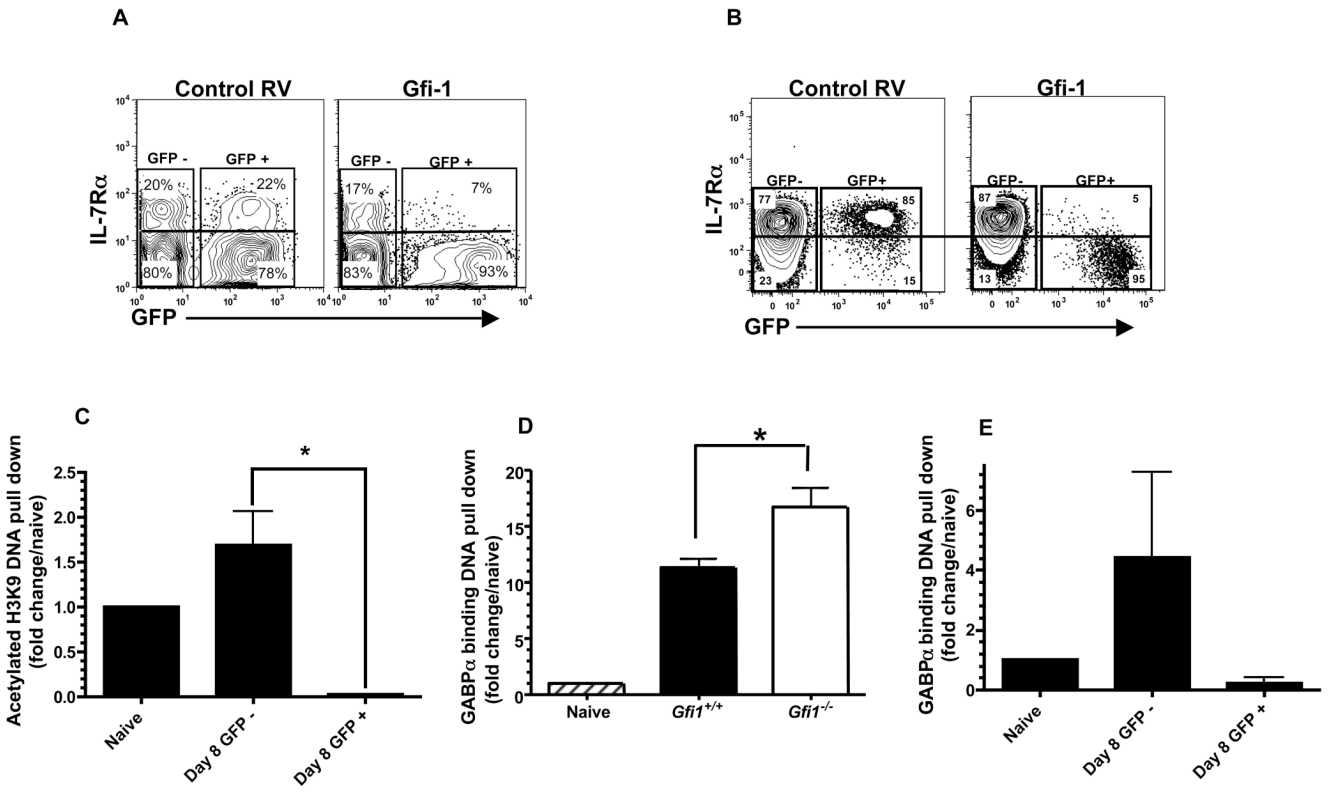

Figure 5. Gfi-1 over expression represses Il7ra by inhibition of GABPα binding to il7rα promoter

A–B. Over expression of Gfi-1 represses IL-7Rα in effector CD8 T cells. Activated P14 CD8 T cells were transduced with RVs (marked by GFP+ expression) expressing Gfi-1 or empty control vector and subsequently transferred into either LCMV infected mice (**A**) or naïve mice (**B**). Seven days later GFP+ P14 CD8 T cells were analyzed for IL-7Rα expression using flow cytometry. Contour plots are gated on donor P14 CD8 T cells and the percent IL-7Rα^{hi} effector CD8 T cells (either GFP+ or GFP−) are indicated. Note, specific reduction in the frequency of IL-7Rα^{hi} effector CD8 T cells in GFP+ P14 CD8 T cells transduced with Gfi-1 RV. The data are representative of 5 individual experiments.

C. Decreased promoter acetylation with Gfi-1 over expression. Day 8 P14 effector CD8 T effector cells either transduced with Gfi-1 RV (GFP+) or not (GFP−) were purified using FACS and analyzed for acetylated H3K9 histones using chromatin immunoprecipitation followed by qRT-PCR. Effector CD8 T cell samples were compared to naive CD8 T cells set at 1 fold. The data in bar graph show the mean ± SEM (n=3); *=p<0.05.

D–E. Gfi-1 inhibits GABPα binding on the *Il7ra* promoter in effector CD8 T cells. Day 8 Effector P14 CD8 T cells in which Gfi-1 was absent (D) or over expressed (E) were purified using FACS and analyzed for GABPa binding on the *Il7ra* promoter using chromatin immunoprecipitation followed by qRT-PCR. Effector CD8 T cell samples were compared to naive CD8 T cells set at 1 fold. In (**D**), GABPa binding to the *Il7ra* promoter is compared between Gfi1+/+ and Gfi1−/− effector P14 CD8 T cells, and in (**E**) effector P14 CD8 T cells transduced with Gfi-1 RV (GFP+) or not (GFP−) are compared. The data in bar graph show the mean ± SEM (n=3); *=p<0.05.